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Perinatal profile of ventricular overload markers in congenital diaphragmatic hernia

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Abstract

Background: In congenital diaphragmatic hernia (CDH), pulmonary hypertension increases right ventricle (RV) afterload, which could impair heart function and contribute to poor outcome for most affected infants. Nevertheless, the real significance of vascular pulmonary alterations in perinatal hemodynamics is largely unknown. It is defined that ventricular pressure overload induces increased myocardium gene expression of B-type natriuretic peptide (BNP) and components of the renin-angiotensinogen and endothelin (ET)–1 systems. Our aim was to evaluate perinatal myocardium expression of these genes associated with ventricular pressure overload in a nitrofen-induced CDH rat model.

Methods: In the nitrofen-induced CDH rat model, fetuses from dated pregnant Sprague-Dawley rats at 15.5, 17.5, 19.5 and 21.5 days postcoitum as well as newborn pups were assigned to 3 experimental groups: control, nitrofen (exposed to nitrofen, without CDH), and CDH (exposed to nitrofen, with CDH). Myocardial samples collected from the RV and left ventricle (LV) were processed for quantification of messenger RNA (mRNA) of BNP, angiotensinogen, and ET-1.

Results: The perinatal expression of BNP, angiotensinogen, and ET-1 mRNA in the RV and LV of the control group revealed daily changes. During gestation, the expression of BNP and angiotensinogen mRNA underwent significant oscillation compared with control in both nitrofen-exposed fetuses, although we cannot identify significant differences between the nitrofen and CDH groups. After birth, we found a significant increasing expression of all studied genes only in the RV of CDH pups.

Conclusions: Perinatal myocardial quantification of BNP, angiotensinogen, and ET-1 mRNA levels suggests that both nitrofen-exposed and control pups revealed prenatal variations of expression of the studied genes. Moreover, CDH is associated with significant molecular alterations only in the RV after birth.

Despite improvements in perinatal care, the mortality of fetuses and newborns with congenital diaphragmatic hernia (CDH) remains exceedingly high [1]. This mortality seems to be related with severe pulmonary hypoplasia and pulmonary

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hypertension (PH) [2]. Several authors suggested that, even in the absence of congenital heart disease, these infants could experience of some degree of cardiac underdevelopment [3,4]. In previous studies, we demonstrated in the experimental rat model of CDH the absence of left ventricle (LV) hypoplasia [5,6] or myocardium immaturity [7] in CDH fetuses. Nevertheless, the real significance of increased right ventricle (RV) afterload, because of neonatal PH, in heart function is still unknown.

Another question that still persists in CDH is the true significance of PH in fetal hemodynamics and its consequences for the fetal heart. In infants with CDH, pulmonary vascular modifications occur from early stages of prenatal development [8] and assume obvious significance after birth. Nevertheless, their meaning in fetal heart function is uncertain. Because fetal heart function is not easily assessed by ultrasonography, the evaluation of markers of ventricular overload in an experimental model of CDH might provide an appropriate alternative. Several biochemical and genetic markers have been suggested to evaluate ventricular load and function, both in animal models and humans, such as B-type natriuretic peptide (BNP), components of the renin-angiotensin system, and endothelin (ET)−1[9].

B-type natriuretic peptide is a hormone of predominantly ventricular origin produced and released in response to increased ventricular wall stress [10,11]. In recent years, it has emerged as a very sensitive biochemical marker for ventricular dysfunction in heart failure as well as in PH, and its plasmatic level could be used to guide the response to therapy and to predict prognosis [12,13]. During normal fetal rat development, a very intense expression of BNP in the heart from 9.5 days postcoitum (dpc) was demonstrated, with major peaks of expression in stages that coincide with landmarks in heart development [14].

The components of the renin-angiotensin system and their roles in adult cardiac hypertrophy have been well documented [15,16]. In adult hearts, the increased hemodynamic load results in increased levels of angiotensin II that stimulates significant hypertrophy and remodeling of cardiac structure. Recent evidence from in vivo studies indicates that angiotensin II also acts as a growth factor and has a potential role in embryonic, fetal, and neonatal development of the heart [17-19]. In addition, the AT1 and AT2 angiotensin II receptor subtypes are present in the heart and are developmentally regulated [20].

Endothelin 1 is a potent vasoconstrictor peptide derived from endothelial cells that is also produced by cardiac myocytes [21]. Endothelin 1 induces myocardial cell hypertrophy and has potent positive inotropic and chronotropic effects on isolated heart muscle. These actions are mediated by the receptors for ET-1 (ETA and ETB receptors) on the cardiac myocytes [22]. The production of ET-1 in the heart is increased in pressure overload conditions, such as PH [23]. Several studies demonstrated that ET system has an important role in the developing heart, contributing to the formation of anatomical structures such as heart outflow tract and great vessels [24].

Interestingly, it was suggested in several previous studies that natriuretic peptides, angiotensin II, and ET-1 could play a significant role in the PH associated with CDH [25-29]. Moreover, in the rat model of CDH, several authors reported significant modifications in heart expression of components from all these systems. Nevertheless, none of these studies assessed the messenger RNA (mRNA) expression of all these genes in both ventricles throughout gestation [30-32].

The aim of the current study was to evaluate, in the nitrofen-induced CDH rat model, LV and RV mRNA expression of BNP, angiotensinogen, and ET-1, genes previously defined as ventricular overload markers, in an attempt to evaluate the significance of PH in myocardium molecular adaptation during fetal development and transition after birth.

1. Materials and methods

The protocols used in this investigation were approved by the Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health Bethesda, MD, (publication no. 85-23, revised 1996).

1.1. Animal model

Pregnancy was obtained in 22 female Sprague-Dawley rats (225 g; Criffa, SA, Barcelona, Spain) after controlled overnight mating, and the finding of vaginal plug was counted as day 0. At 9.5 dpc, pregnant rats were exposed to 100 mg of nitrofen (2,4-dichlorophenyl-p-nitrophenylether) dissolved in 1 mL of olive oil administered by gavage or to an equal volume of olive oil alone [33]. Randomly, fetuses, treated with nitrofen or olive oil, were harvested by cesarean section at 15.5, 17.5, 19.5, and 21.5 dpc (term gestation, 22 days), weighed on a precision balance (SBC 21; Scaltec Instruments, Heeligeesdadt, Germany), and killed by decapitation. To evaluate newborn rats, the gestation was continued in some pregnant rats until 22 dpc, and rats were allowed to deliver spontaneously. Newborns rats were harvested immediately after death or electively killed, by decapitation, at 6 hours after delivery. In this regard, it might be emphasized that all CDH pups died before our set point. After weighing pups, laparotomy was performed under binocular surgical microscopy (Wild M651.MS-D; Leica, Herbrugg, Switzerland) to inspect the diaphragm. Hearts were excised en bloc through median sternotomy. Myocardial samples were harvested from LV and RV free wall and snap-frozen at −80° for further molecular studies. Fetuses were assigned to the following 3 experimental groups: (i) control group—fetuses or pups exposed to olive oil alone, without CDH; (ii) nitrofen group—fetuses or pups exposed

to nitrofen without CDH; and (iii) CDH group—fetuses or pups exposed to nitrofen with CDH. Fetuses and pups with structural cardiac defects were excluded. Because it was not feasible to accurately identify a diaphragmatic defect at 15.5 dpc, only 2 groups were defined: control and nitrofen (exposed to nitrofen with or without CDH).

1.2. Molecular studies

1.2.1. Ribonucleic acid extraction and reverse transcription

Total mRNA from LV and RV samples of 150 fetuses (n = 10 each for control, nitrofen, and CDH, in each studied time-point: 15.5 dpc, 17.5 dpc, 19.5 dpc, 21.5 dpc, and newborn) was extracted using the RNeasy Mini Kit Protect (74712; Qiagen, Hilden, Germany). Quantification of total mRNA was done by spectrophotometry (BioPhotometer, Eppendorf, Germany), and the A260/A280 ratio was used to test protein and deoxyribonucleic contamination of the extracted product.

Reverse transcription was performed as previously described by Santos et al [34].

1.2.2. Quantitative polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed as previously described by Santos et al [34].

Primer design was based on the available sequences in GenBank (NCBI-NLM-PubMed-Gene). All the primers are intron spanning (Table 1). For all the primer sets, standard amplification curves (ST curves) were made with randomly selected complementary DNA samples, setting $r = 0.99$. The samples gene's expression was normalized for β-actin.

1.3. Statistical analysis

Results were presented as mean ± SEM. Statistical analysis was performed using the SigmaStat 3.5 software (London, England). The different data sets of control, nitrofen, and CDH groups failed in the Kolmogorov-Smirnov test for normality. Therefore, statistical analysis was performed by the 1-way analysis of variance on ranks and the Dunn test for posttest analysis. Statistical significance was set at $P < .05$.

2. Results

The mRNA levels of BNP, angiotensinogen, and ET-1 (through gestation, in the RV and LV, in the control, nitrofen, and CDH groups) are presented in Figs. 1–3, respectively.

The BNP mRNA expression in the RV demonstrated in control group a significant decrease of around 19.5 dpc, followed by a peak increase at 21.5 dpc. After birth, its levels are similar to earlier studied stages of heart development. In comparison with the control group, we found significant differences both in nitrofen and CDH groups, characterized by a mirror-image pattern of expression, with inverse peak increase at 19.5 dpc and a subsequent decrease at 21.5 dpc. After birth, the BNP mRNA expression in RV was similar in the nitrofen and control groups, but in the CDH group, we found a significant increase in its expression (5.5-fold) (Fig. 1).

The BNP mRNA expression in LV revealed a similar expression pattern in all study groups during fetal development. The control group had a significant decrease of around 19.5 dpc followed by an increase at 21.5 dpc, although its magnitude was not as evident as occurred in the RV. Regarding the nitrofen and CDH groups, we also found a mirror-image pattern of expression. Nevertheless, after birth, the levels of BNP mRNA were similar to prior stages of heart development in the 3 groups (Fig. 1).

Concerning the expression of angiotensinogen mRNA in the RV, we observed a slight increase from 17.5 dpc that peaked at 19.5 dpc, followed by a decrease to basal levels at 21.5 dpc. After birth, its levels remained constant. In the nitrofen and CDH groups, there was no significant variation in angiotensinogen mRNA expression through gestation. At birth, a significant rise occurred in the CDH group, compared with the control and nitrofen groups (Fig. 2).

Similar to the expression of BNP, in the control group, we found an LV angiotensinogen mRNA expression pattern similar to RV. Its expression rose from 17.5 dpc, peaked at 19.5 dpc, then slowly decreased until birth. Regarding the nitrofen and CDH groups, angiotensinogen mRNA levels in LV did not vary during gestation and after birth (Fig. 2).

In the control group, ET-1 mRNA level in RV showed a significant peak of expression at 19.5 dpc, followed by a constant level of expression that persisted even after birth. During fetal development, both the nitrofen and CDH groups

Table 1 Primers used for quantitative polymerase chain reaction

Gene	Accession no.	Primer set	Product size (base pair)
BNP	NM_031545	5′-GCA GAA GCT GCT GGA G-3′ 5′-GCT GTC TTG AGA CCT AAG GA-3′	118
Angiotensinogen	NM_134432	5′-GGATAAGTCCAGAGAGCGAG-3′ 5′-CAGACACCCCTGCTACAGTC-3′	129
ET-1	NM_012548	5′-CAGAAACAGCTGTCTTGGGA-3′ 5′-GGAGGAGCAGGAGCAACG-3′	116
β-actin	NM_031144	5′-GAT TTG GCA CCA CAC TTT CTA CA-3′ 5′-ACT TTG GTC ATC TTT TCA CGG TTG G-3′	114

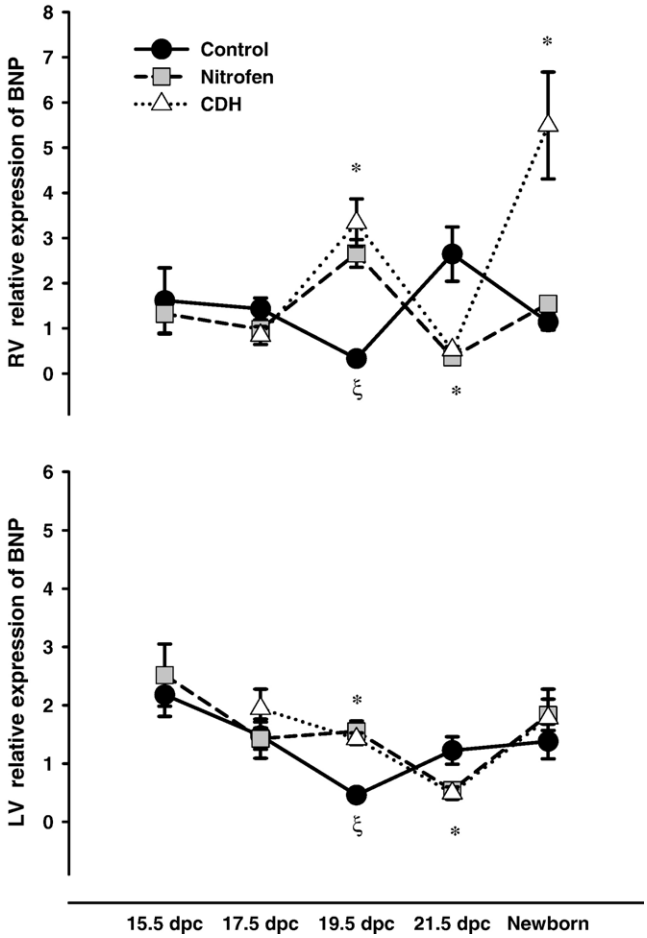

Fig. 1 Expression of BNP mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ vs 16 dpc.

had exactly the same expression pattern, without any significant difference from control. However, after birth, whereas in the nitrofen group, we did not found any difference from the control group, the CDH group had a significant increase in ET-1 mRNA expression (Fig. 3).

Finally, the LV the expression of ET-1 mRNA showed a peak at 21.5 dpc and returned to baseline levels after birth. In the nitrofen and CDH groups, we found the same pattern of expression of the gene, without any difference compared with the control group (Fig. 3).

To evaluate the relative expression changes of mRNA of studied genes in the LV and RV after birth, we used the RV-to-LV mRNA ratio (Fig. 4). In the control group, mRNA of the 3 genes had similar relative expression in both ventricles. On the other hand, in the CDH group, we found a very significant increase in the RV-to-LV ratio expression patterns of BNP, angiotensinogen, and ET-1.

3. Discussion

In this study, we determined the cardiac expression of BNP, angiotensinogen, and ET-1 mRNA during perinatal development in normal and nitrofen-exposed rats. Cardiac expression of these genes showed temporal changes, suggesting a closely regulated developmental expression. Our results also showed late fetal cardiovascular disturbances in nitrofen-exposed fetuses. Moreover, it clearly illustrates that CDH pups, in early postnatal adaptation, experience severe RV molecular adaptation to pressure overload.

In our day, CDH remains a challenge in perinatology. The most severely affected babies have an extremely high mortality rate despite aggressive treatment. Persistent hypoxia because of pulmonary hypoplasia and hypertension is considered by several authors as the main problem in CDH. Nevertheless, centers without extracorporeal membrane oxygenation did not significantly improve the outcome using techniques targeted to lung-dependent oxygenation, such as high-frequency ventilation or pulmonary vasodilator therapy. Although the real significance of PH is not completely defined in infants with CDH, it is well known that severe PH is associated with increased mortality

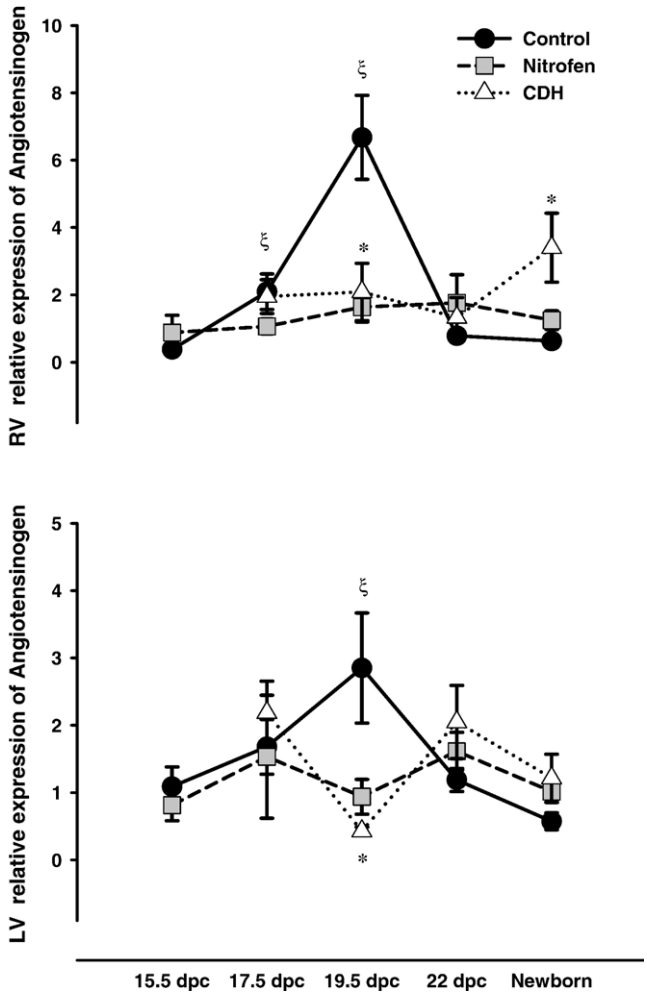

Fig. 2 Expression of angiotensinogen mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ 16 dpc.

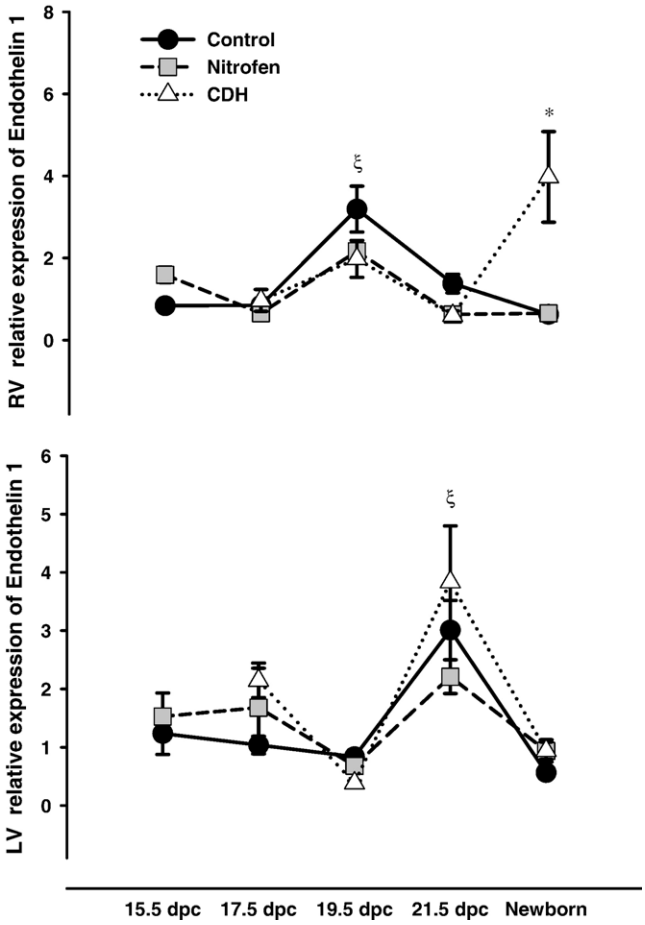

Fig. 3 Expression of ET-1 mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ vs 16 dpc.

[35]. Pulmonary hypertension interferes with gas exchange as well as hampers myocardial performance, with additional compromise of pulmonary blood flow and tissue oxygenation [36].

From fetal to adult life, cardiopulmonary interaction plays a significant role in physiologic hemodynamics. Pathologic conditions affecting the lung or the heart could hamper this equilibrium. Although the pulmonary consequences of cardiac disease are well recognized, the influences of pulmonary changes on cardiac function are less well appreciated, particularly in fetuses. In late fetal life, several physiologic hemodynamic changes occur, such as initiation of ductus arteriosus closure and pulmonary vasodilatation, as well as increase in RV output to the pulmonary artery [37]. These modifications are promoted by many paracrine factors, among which systems such as natriuretic peptides, angiotensin, and ET-1 are supposed to play a role in different stages of heart development. Although significant alterations in these systems are well documented in pressure and volume cardiac overload in adults, the exact expression pattern of these genes in relation to fetal cardiac load was not previously defined. In our study, we demonstrated a distinctive cardiac expression pattern of these genes during normal perinatal fetal rat development, presumably related to hemodynamic variations.

Endothelin 1 mRNA expression is different in the LV and RV, probably because of different loads in the LV and RV. This gene has a peak expression at 19.5 dpc in the RV, whereas in the LV, this occurs at 21.5 dpc. On the other hand, angiotensinogen and BNP have a very similar expression pattern in the RV and LV but a mirror expression compared with each other. We established that, in both ventricles, when BNP increases, angiotensinogen decreases. The opposed effect of natriuretic peptides and angiotensin in myocardium as well as in vessels is well known. Generally, BNP has potent inhibitory effects on the renin-angiotensin-aldosterone system [38], and in heart failure, activation of the renin-angiotensin-aldosterone system is suppressed by BNP [39]. These systems might form an important regulatory complex of fetal vascular physiology and development. Hypothetically, dysregulation of these delicate control mechanisms could interfere with the cardiopulmonary hemodynamics and lead to disease.

Pulmonary vascular abnormalities in CDH have been well described from early stages of lung development. They consist of fewer pulmonary arteries per unit lung volume and peripheral muscularization of small arteries with medial and adventitial thickening [8]. Although the underlying mechanisms are not completely understood, these anomalies may cause abnormal vascular reactivity, and CDH lungs may become unable to adapt normally at birth [40]. The importance of abnormal vascular development as a determinant of survival in CDH has just recently been appreciated. Nonetheless, we are far from understanding the specific interplay of the factors regulating vascular tone in CDH as well as the significance of those anomalies during fetal heart and lung development.

In our work, we intended to define the fetal and neonatal expression pattern of 3 genes related to ventricular pressure

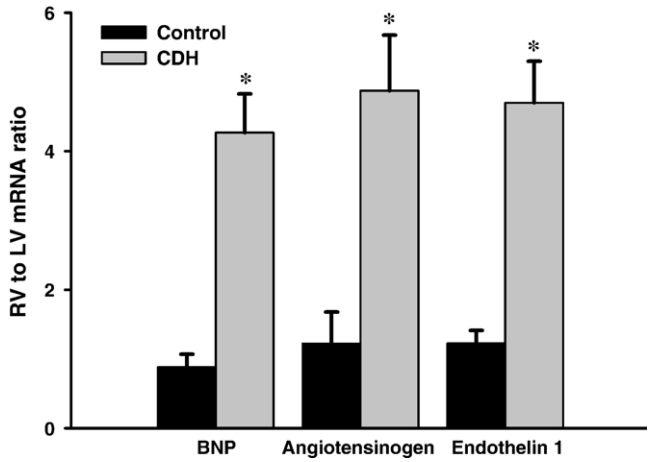

Fig. 4 Right ventricle–to–LV mRNA ratio of BNP, angiotensinogen, and ET-1 in control and CDH groups at 6 hours after birth. *$P < .05$ vs control group.

load in the nitrofen rat model of CDH. We demonstrated that during late fetal life, the nitrofen-exposed fetuses had significant variations in heart expression of mRNA BNP and angiotensinogen, evocative of cardiovascular disturbances. Remarkably, the mirror expression pattern of these 2 genes observed in control rat fetuses is preserved in nitrofen-exposed fetuses. Nevertheless, there are no alterations in ET-1 mRNA cardiac expression, and the variation reported in angiotensinogen and BNP genes occurred both in CDH and non-CDH nitrofen–exposed fetuses. These results suggest that the abnormalities observed are probably consequence of nitrofen action and not related to a hypothetical pulmonary vascular remodeling. We believe that, as occurs in several congenital malformations, PH in CDH is balanced during fetal life and should not have hemodynamic consequences or induce cardiac adaptation. In fact, pulmonary vascular remodeling, when present, should not cause elevated RV pressures in the fetus given the presence of the ductus arteriosus.

In infants with CDH, intrauterine pulmonary hypoplasia and vascular remodeling may cause failure of pulmonary vascular resistance to fall at birth. This event implies an increased pressure overload to RV, with additional wall and endothelium stress, responsible for the initiation of cardiac adaptation. Several studies suggest that in PH, the LV experiences both systolic and diastolic function adaptation because of bulging of the ventricular septum and diminished RV output [41]. In our study, the increased levels of all studied cardiac pressure overload markers in the RV of CDH pups after birth may indicate increased pulmonary vascular resistance in the CDH group. This response is specific for RV, and in this model, we did not demonstrate any LV molecular adaptation to PH.

In conclusion, perinatal myocardial quantification of BNP, ET-1, and angiotensinogen mRNA demonstrated that CDH is associated with significant molecular adaptation only in RV after birth. In fact, although nitrofen induced a hemodynamic imbalance in the expression of these genes, the major and novel observation from our work is the absence of cardiac impact of PH during late fetal life.

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